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13. ABSTRACT ***(Maximum 200 Words)***

This research project was aimed at finding new, more efficient and effective ways to detect, identify, and study biomolecules. The approach to be taken is to start with fluorescent nucleoside-like molecules in which the DNA base is replaced with known hydrocarbon and heterocycle fluorophores. These molecules are termed "fluorosides", short for "fluorescent deoxyribosides". They can be assembled on a DNA synthesizer into any desired number and order, both alone and alongside natural DNA bases.

The specific aims of the project were (A) to find enzymes that can label biomolecules with excimer-forming fluorophores, resulting in labels that are brighter and have longer Stokes shifts than the current standard; (B) to make excimer- and exciplex-forming probes for repetitive DNA sequences such as telomeres and centromeres, and to demonstrate them both outside and inside cells; and (C) to make novel polyfluorophore molecules that can sense many different types of small molecules, both in solution phase and in the vapor phase.

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FINAL PROGRESS REPORT

GRANT # DAAD19-03-1-0066

TITLE: **Use of Multiple Fluorescent Labels in Biological Sensing**

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Table of Contents

BODY OF FINAL REPORT

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GRANT # DAAD19-03-1-066

TITLE: **Use of Multiple Fluorescent Labels in Biological Sensing**

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INTRODUCTION

This report covers the total award period for this grant. This project has been ongoing at Stanford University for three years (2003-2006). The chief aims of the project were:

Specific Aims of this project:

(1) to find enzymes that can label biomolecules with excimer-forming fluorophores, resulting in labels that are brighter and have longer Stokes shifts than the current standard.

(2) to make excimer- and exciplex-forming probes for repetitive DNA sequences such as telomeres and centromeres, and to demonstrate them both outside and inside cells.

(3) to make novel polyfluorophore molecules that can sense small molecules, both in solution phase and in the vapor phase.

FINAL TECHNICAL PROGRESS REPORT

The long-term goals of this project were to study the basic science of how multiple fluorescent molecules interact when placed near one another, and to apply this information in the design of new molecular sensing platforms, with applications in detection of DNA, RNA, physical properties in solution, and small molecules and ions. DNA was used as a scaffold to arrange the fluorescent molecules in close proximity.

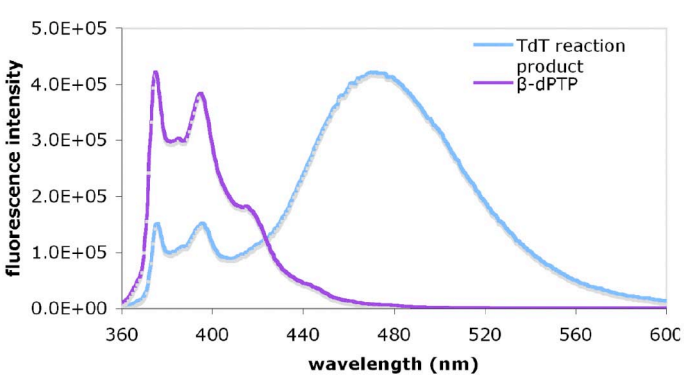
The project as a whole enjoyed considerable success in several areas of basic and applied science. We studied several modes of interaction between fluorophores, and characterized unusual interactions, including water-soluble excimers and exciplexes. We investigated multiple ways to assemble poly-labeled molecules, including organic synthesis and enzymatic synthesis. Finally, we

succeeded in developing different classes of molecules that can sense diverse metal ions in solution, some that sense photons, and some that detect RNAs in living cells. In total, seventeen papers were published and two more were submitted for publication.

AIM 1: Finding enzymes that label DNAs with excimer-forming fluorophores

An important part of our program was the exploration of the possibility of using DNA polymerase enzymes such as terminal deoxynucleotidyl transferase (TdT) to assemble non-natural fluorescent oligomers. This was done for two chief reasons: efficiency and ease of use. In terms of efficiency, polymerase enzymes can operate with 99.99% yields, giving polymers thousands of nucleotides long in less than a minute. Moreover, enzymes can work on extremely small scales, working routinely with nanomolar DNA concentrations, and picomolar quantities, while DNA synthesizers commonly make much larger quantities of DNA and require liters of organic reagents and solvents. As a result, not only are enzymes cheaper and more efficient, but they are environmentally friendly ("green") as well, requiring only water and producing nontoxic byproducts. As for our interest in ease of use, enzymes are routinely used in a much greater range of laboratories by a much greater number of people with interests in biochemistry, biology, and medicine than are DNA synthesizers, which require synthetic chemistry knowledge and training. Since many of the long-range applications of our molecular sensing approaches will be biological and biomedical, it makes sense to enable the scientists who will be applying the technologies to be able to make the probes themselves when needed.

Our research during this grant period was successful in developing the TdT enzyme for synthesis of nonnatural DNA-like polyfluorophores (see figure). Our research determined that standard hydrogen bonding nucleotides are not required for this enzyme, and that nonpolar nucleoside isosteres could be very efficiency substrates, being incorporated dozens of times into new polymers at the end of DNA oligonucleotides. In addition, we demonstrated that expanded DNA nucleotides could also be efificent substrates. These are of special interest because they are inherently fluorescent nucleotides that bind natural DNAs and RNAs very tightly.



Enzymatic synthesis of pyrene nucleotide oligomers by TdT. Emission spectra of oligomeric product (blue) compared with starting nucleotide (violet).

Finally, we published a paper demonstrating that simple aromatic hydrocarbons (specifically pyrene in this case) could be substrates as the deoxynucleoside triphosphate derivatives. The resulting

short oligomers showed efficient excimer fluorescence. Thus the reaction showed a clear change in fluorescence emission, from violet-blue (the emission of pyrene monomer) to green (pyrene excimer). Not only was this result of interest as a demonstration of how to label DNAs with excimer dyes; it also raises the interesting possibility of new solution-phase assays for apoptosis.

AIM 2: Testing excimer-forming fluorophores for labeling repetitive sequences of DNA.

In this Aim we studied several designs for DNA probes that (a) bind repetitive DNA sequences and (b) yield excimer signals. To carry this out, we studied the above-mentioned pyrene nucleoside as a prototype, since it was known to form excimers when synthesized into oligomers where pyrene was directly adjacent. The challenge of this Aim was then to design DNA probes that used single pyrene nucleotides at teir ends. If designed correctly (we hypothesized), such probes would bind side-by-side on repeating DNA targets; this would bring the pyrene labels into adjacent positions, potentially yielding an excimer signal. Thus unbound probes and bound probes would in principle yield a color change in solution. If successful, this could give probes of repetitive sequences in solution and in cellular systems. Repetitive sequences have special biological properties; one important example is the telomere, a repeating hexamer sequence that exists in hundreds of copies at the ends of human chromosomes. Telomere sequence, length and structure is associated with a number of human diseases, so probing these sequences could have useful applications in biomedical science.

Unfortunately our attempts to date have so far failed to give useful signal changes, for two interesting reasons. First, we found, interestingly, that labeling a DNA probe with one pyrene at each end gave a relatively strong pyrene excimer signal. This was the case even for relatively long oligomers of 12 nucleotides tagged with two pyrenes. This suggests that pyrenes are either self-associated in the oligomers (meaning presumably that the probes exist in a ring-like shape, with the ends binding each other), or that they can associate rapidly with one another in the excited-state lifetime of pyrene. Of course, this observation prevents a strong change in signal on binding, since excimer was already seen prior to binding.

A second problem with this approach appeared to arise from relatively strong internal structure of the repetitive sequences. Telomeric sequences can fold (usually in the presence of potassium ion) into 4-stranded structures. It was our hope that these structures would be relatively weak in the absence of potassium, and that oligomers would bind better as Watson-Crick complements, displacing this structure. However, we noted that binding of probes to these sequences appeared to be weak, even when DNA modifications known to increase affinity were used. This suggests that the internal folding of telomere sequences may be stronger than commonly thought.

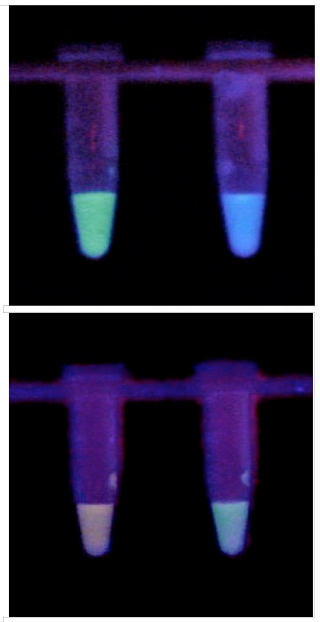
Future experiments with this aim might benefit from molecular strategies that could prevent self-association of the excimer-forming dyes, and from use of even tighter-binding DNA modifications.

AIM 3: Study of novel polyfluorophore sensors.

Our long-term goal in this Aim was to take advantage of the fact that DNA-like oligomers of fluorophores act cooperatively to yield new fluorescence properties as an interacting electronic unit. Thus, changing the environment of some of these oligomers might yield substantial changes in fluorescence, depending on the specific natural of the interaction of the monomeric chromophores. Our studies in this area were quite successful.

In the first test of sensing by oligomeric fluorophores built on a DNA backbone, we studied the response of a large library of tetramers (nearly 15,000 molecules) to light exposure. Our strategy for identifying sensors that respond to a stimulus is to use digital images of the library before and after the stimulus to compare changes in individual beads. We found that some members of the library responded in very different ways to UV light exposure. For example, some of the molecules were resistant to photobleaching, which is a useful property of dyes that could be applied to biological studies. This early observation will be pursued in future studies.

Remarkably, we also found that some dyes were extremely sensitive to light exposure, losing their original emission in minutes to seconds under a standard mercury lamp on the microscope (see figure at right). When we identified, synthesized, and re-studied these individual cases, we found that many of them did not simply photobleach, which implies photostimulated reaction with oxygen to destroy the fluorescence altogether. Instead, we found that these unusual molecules changed color: from red to blue, green to blue, and other changes. Thus these molecules can act as quite sensitive reporters of light exposure. Such reporters could be applied to biological systems in many different ways. One example is the real-time imaging of movements and diffusion of local molecules in a specimen.



Two examples of color- changing sensors of light exposure. At left is before exposure, at right, after exposure. Molecules are DNA-like tetramers of fluorophores, having specific sequences that were identified from a 14,000 member library.

In a new extension of this Aim, we reported on the design and synthesis of fluorescent DNA base replacements that bind and sense metal ions. Two pyridoimidazole ligands designed to bind metals having affinity for nitrogen were prepared. In a recently published study, the monomer nucleosides of these compounds were shown to be fluorescent, and to change fluorescence in the presence of metal ions. Changes observed include quenching of emission, enhancement of emission, and shifts in wavelength of emission, depending on the metal ion. We expect

that in the future, oligomers containing such species might act as diverse sensors of many different metal ions in solution.

Additional results derived from multi-fluorophore studies

Our research on multi-labeled modified DNAs yielded useful information in several applications of sensing. One of these not explicitly included in the above aims was detection of RNAs in living cells. We developed probes that contained one quencher and up to two other fluorophores, and used them as color-changing indicators of ribosomal RNAs, and later of messenger RNAs, in cellular systems. The probes were exceedingly simple to use, requiring only 1-2 h of incubation with the cells, followed by analysis of the results. This analysis involved only looking at them under the microscope, or automated analysis by flow cytometry. This work was assisted by knowledge and support from the ARO-funded research, and a number of published papers acknowledged this. This research could have direct application in sensors of pathogenic agents.

KEY RESEARCH ACCOMPLISHMENTS, 2003-2006

• We characterized an enzyme that can synthesize nonnatural fluorescent DNA-like oligomers. These oligomer tags are added to the ends of natural DNAs by this enzyme, resulting in bright excimer fluorescence, yielding a color change in solution.

• We observed multiple classes of energy transfer in fluorescent oligomers, and characterized properties (such as enhanced brightness and large stokes shifts) that are not available in commercial fluorescent labels.

• We discovered DNA-like fluorescent oligomers that can act as sensors of light exposure.

• We developed new nucleoside compounds that can act as fluorescent sensors of metal ions.

• We developed new classes of multi-labeled DNA probes that can detect and identify RNA sequences in living bacteria, including pathogenic strains.

REPORTABLE OUTCOMES

Nineteen papers were published during the grant period. Electronic copies were sent previously for 1-17. Pdf copies of #18 and #19 are attached.

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DOI: 10.1002/cbic.200500515

Enzymatic Synthesis of Fluorescent Oligomers Assembled on a DNA Backbone

Younjin Cho and Eric T. Kool*[a]

A number of research laboratories have investigated the properties of multichromophore molecules and their applications in materials science and in biotechnology. Previous approaches for preparing such molecules have involved traditional organic synthesis. Here we describe the one-step enzymatic synthesis of such a multichromophore species by using a DNA-polymerizing enzyme (terminal deoxynucleotidyl transferase (TdT)). We find that a nucleotide-like molecule with pyrene replacing the DNA base (dPTP) can be accepted as a substrate for this enzyme to produce discrete chromophores that have 3 or 4 pyrenes consecutively, depending on which anomer (α or β) is used. Products were characterized by gel electrophoresis, mass spectrometry, and fluorescence. The reaction was found to change the fluorescence emission of the chromophore from a maximum at 375 nm (the monomer nucleotide) to 490 nm in the oligomeric product. This new green–white emission is consistent with the formation of a pyrene excimer between adjacent pyrene glycosides, which exhibit a large Stokes shift of 130 nm. The enzymatic synthesis of the pyrene excimer might have applications in homogeneous biological assays for DNA fragments, such as those that arise during apoptosis.

Introduction

Recent studies have shown that molecular assemblies of multiple chromophores can be useful in light-harvesting and energy-transfer applications, and in amplifying signals in biological sensing. Examples of scaffolds for bringing together multiple light-absorbing and -emitting species include dendrimers,[1] peptide backbones,[2] hydrocarbon frameworks,[3] organic polymer backbones,[4] and DNA.[5] In the last category, we have prepared DNA-like oligomers in which DNA bases are replaced by fluorescent chromophores.[5a,b,6] Because of their closely stacked structures, these DNA-like species display multiple energy-transfer modes such as excimers, exciplexes, and fluorescence resonance energy transfer.[5a] Such multichromophoric species might have useful applications in molecular detection and in sensing; for example, a recent report described a combinatorial library of tetrameric fluorophores, and identified molecules in the library that could sense light exposure, resulting in discrete color changes.[5b]

Although the various classes of chromophore assemblies have shown promising properties, to date all of them have been prepared by classical organic synthesis methods. We envisioned that an alternative approach to the preparation of such species—namely, enzymatic synthesis–might be possible, especially for those assemblies that rely on natural backbone structure. Enzymatic synthesis offers a number of possible advantages, including mild conditions, high yields for multiple steps, applicability on small biological scales, and ease of application in biomedical laboratories. Here we describe the use of a template-independent DNA polymerase for the synthesis of oligomeric pyrene assemblies using the DNA backbone.

Results

Terminal deoxynucleotidyl transferase (TdT; EC 2.7.7.31) is a template-independent X-family DNA polymerase that catalyzes the random addition of nucleotides to the 3′-hydroxyl group of a DNA strand.[7] Its biological role is to increase the diversity of the immune repertoire by adding random nucleotides, called N regions, at gene segment junctions during V(D)J recombination.[8,9] TdT is useful for labeling the 3′-end of DNA, usually employing radiolabeled natural nucleotides or modified uridine nucleotides conjugated with fluorescein or other reporter groups.[10]

We employed considerably larger and non-natural pyrene deoxyribosides[11] in our enzymatic studies (Scheme 1). Previous experiments have shown that T7 and Kf DNA polymerases could accept the β anomer of this unusual nucleotide analogue (β-dPTP) as a substrate, but they only incorporated a single monomer, probably in part because of unfavorable enzyme interactions with the DNA template.[12] However, TdT does not require a template strand to polymerize DNA.[7] Thus we hypothesized that it might be possible to incorporate more than one pyrene nucleotide successively by using TdT, as there might be fewer hydrogen-bonding requirements in the enzyme or distortion of DNA duplex structure to consider.

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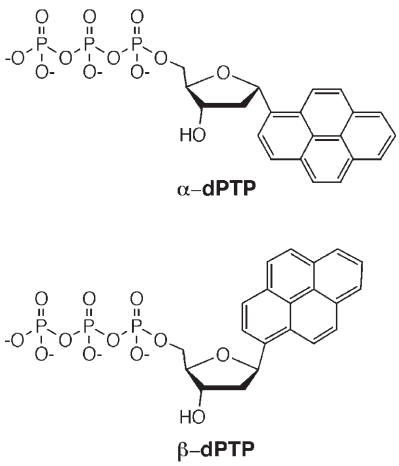
Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author.

Both anomers of pyrene deoxyriboside were prepared as described previously.[11,13] Ludwig's procedure was then used to convert both α and β anomers of the pyrene nucleoside to the 5′-triphosphate derivatives.[12a,14] The α anomer triphosphate derivative was previously unknown. We chose to test both anomers because there were previous literature reports in which α anomers of natural nucleosides and their analogues showed different substrate activities from β isomers with DNA polymerases.[15] Fluorescence spectra in water revealed that the monomeric nucleotides have similar fluorescence characteristics to pyrene, emitting blue fluorescence (emission maximum = 375 nm with excitation at 345 nm; Figure S1, Supporting Information) with a quantum yield of 0.25 for α-dPTP and 0.34 for β-dPTP.

Using the homooctamer of thymidine (dT_8) as a primer, we studied pyrene nucleotide incorporation into DNA by TdT, monitoring extension products by gel electrophoresis. The results showed that both β-dPTP and the previously unknown (and more unnaturally configured) α-dPTP acted as substrates of TdT, and multiple pyrene nucleotides could be inserted (Figure 1). Interestingly, there was a limit to the number of pyrene residues incorporated consecutively. TdT stopped after three pyrene nucleotides were added for the α anomer of dPTP or four for the β anomer (Figure 1). Additional quantities of enzymes and nucleotides did not yield further extension (Figure S2, Supporting Information). Thus, unlike TdT extension with natural thymidine triphosphate (and its conjugates), in which a broad range of polymeric products is produced, the oligomeric pyrene extension produced discrete products. In the optimum case, β-dPTP produced a tetrameric adduct as the dominant product (Figure 1A, lane 17). Products were characterized by MALDI-mass spectrometry, which showed the addition of the mass of three and four pyrenes to the dT_8 primer (Figure S3, Supporting Information). The pyrene-elongated products were purified by size-exclusion chromatography and HPLC. Analysis of the enzymatic reaction product showed clear evidence of green–white excimer emission from the β-pyrene oligomers with long-wavelength UV excitation (Figure 2), as was also observed with authentic material prepared previously by total synthesis (Figure S4, Supporting Information). The α-pyrene oligomers have recently been shown to give very similar excimer emission.[18] The difference in color and intensity between the oligomeric product and the starting monomer at the same concentration is clearly visible to the eye (Figure 2A). To test whether the extended products could act as substrates for further extension, additional dPTP and additional enzyme were added after the original 1 h incubation. However, no further dNTP extension occurred after the pyrene insertion (Figure S2); this suggests that some structural or physical property of the oligomeric



Scheme 1. Structures of the α and β pyrene nucleoside triphosphates.

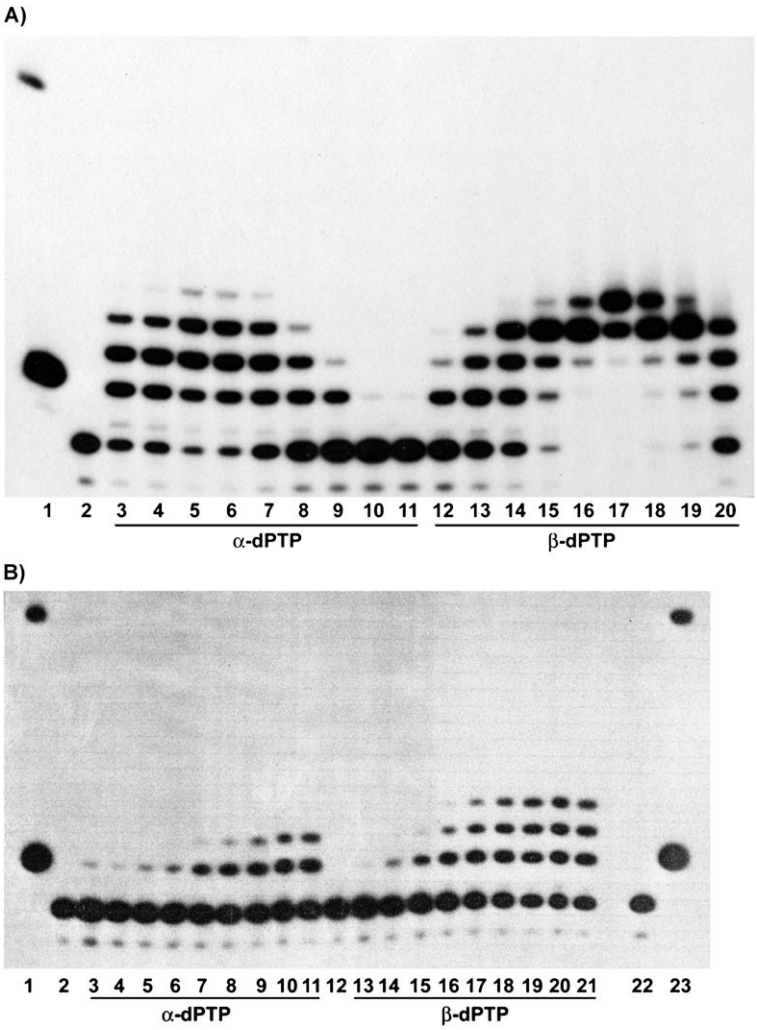


Figure 1. Autoradiograms of polyacrylamide gels showing TdT insertion of pyrene nucleoside triphosphate into DNA primers. A) Concentration dependence study of dPTP incorporation by TdT. Lane 1: 10 bp size marker, lane 2: primer only, lanes 3–11: TdT reaction with increasing concentrations (2.5–640 μM) of α-dPTP, lanes 12–20: TdT reaction with increasing concentrations (2.5–640 μM) of β-dPTP. B) Time course of dPTP incorporation by TdT. Lanes 1 and 23: 10 bp size marker, lanes 2 and 12: primer only, lanes 3–11: TdT reaction with α-dPTP with increasing incubation time (10 s–50 min), lanes 13–21: TdT reaction with β-dPTP with increasing incubation time (10 s–50 min), lane 22: primer with TdT (control).

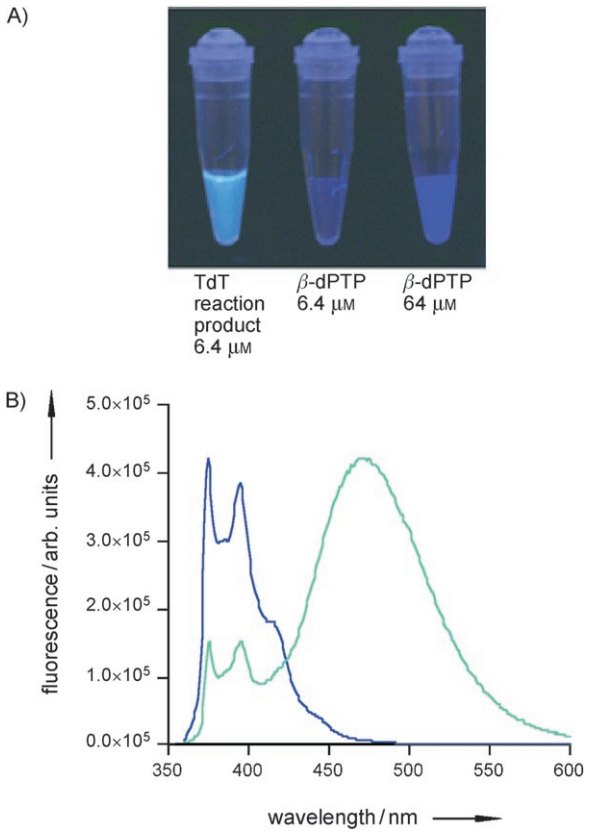


Figure 2. Fluorescence of the purified TdT reaction product with β-dPTP. A) Comparison of the bright greenish-blue fluorescence of the single-stranded TdT reaction product to the violet fluorescence of β-dPTP under UV excitation. B) Normalized emission spectra of the TdT reaction product (green) and β-dPTP (blue) in water (excitation at 345 nm).

pyrene tails causes them to cease as substrates once they are elongated to their characteristic lengths.

Discussion

In the large majority of literature reports, TdT is used with natural nucleotides or natural nucleotides conjugated with fluorophores, such as fluorescein-dUTP, to produce polymeric tails.[10a] Only one previous example is known of a non-natural nucleobase substrate for TdT, 5-nitroindole nucleotide,[10a] which was studied as a monomer for antibody tagging.[16] With TdT, 5-nitroindole nucleotide was incorporated in a broad range of lengths, with 7 to >100 monomers being incorporated.[10a] No fluorescence studies were reported. Presumably the production of only short, discrete products in the present study is a result of the larger, more hydrophobic and less natural structure of the pyrene nucleotides. Regardless of the reason, this makes it possible to easily generate relatively homogeneous excimer labels without risk of over-extension by too-long incubation. In this regard, the short, defined nature of the tri- or tetrapyrene tags is a useful outcome, rendering the products more chemically homogeneous and easier to purify, and keeping them well soluble in aqueous solution.

To our knowledge, this is the first example of excimer formation by an enzymatic reaction. Pyrene excimer has been studied in the context of DNA by several laboratories.[17] It has potential advantages as a fluorescent reporter over fluorescein, one of the most commonly used commercial fluorophores in TdT labeling. Pyrene excimer exhibits incremental emission intensity increases with the number of pyrene residues; this leads to improvement in the detection sensitivity, whereas fluorescein is self-quenched with multiple adjacent DNA substitutions.[18] Moreover, pyrene excimer has a large Stokes shift of 130 nm, compared to about 20 nm for fluorescein. This large separation between excitation and emission further increases sensitivity, by avoiding interference from the excitation light source. Significantly, there is a large color change from violet to green on oligomerization of pyrene monomers; this suggests the possibility of homogeneous labeling and assays that would not be possible with fluorescein. The results also suggest the possible use of oligomeric chromophores such as tetrapyrene for in situ assays with TdT such as TUNEL (TdT-mediated dUTP Nick-End Labeling) assays for apoptosis,[19] and a biochemical TdT activity assay for leukemia diagnosis.[20]

Our study has demonstrated that multiple pyrene residues can be incorporated into DNA by TdT, by using both α and β anomers of a non-natural pyrene deoxyriboside. The surprising acceptance of these large, non-natural nucleotides as polymerization substrates suggests the possibility of other designed species being accepted as well. Future work will be aimed at testing other unusual substrates, and at developing applications of excimer fluorescence that results from such polychromophoric systems.

Experimental Section

Standard conditions for TdT insertion of pyrene nucleoside triphosphate into DNA primers: Primers were 5′-end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase and purified by using MicroSpin™ G-25 columns (Amersham Bioscience). Insertion reactions were carried out in the presence of Tris-acetate (pH 7.9, final concentration 20 mM), potassium acetate (50 mM), magnesium acetate (10 mM), $CoCl_2$ (0.25 mM), and dithiothreitol (1 mM). An aliquot of labeled primer (4 pmol) was mixed with TdT (recombinant; New England Biolabs; 25 units), and the solution was incubated for 2 min at 37 °C. Reactions were initiated by adding the primer/enzyme cocktail (10 μL) to an equal volume of a dPTP or dNTP solution (200 pmol). The reactions were run for 1 h at 37 °C and then quenched by being heated to 90 °C for 5 min. For the concentration-dependence studies, the preincubated primer/enzyme cocktail was mixed with various concentrations of dPTP (2.5–640 μM). For the time-course studies, the reactions were quenched after the designated time (10 s–50 min). Reaction products were resolved from unreacted primer by electrophoresis on 20% denaturing polyacrylamide gels and visualized by autoradiography.

Purification and characterization of products: The reactions were run under the standard conditions for TdT elongation, except that unlabeled primer and a larger amount of β-dPTP (400 pmol) were used. The pyrene-elongated products were separated from the unincorporated β-dPTP and primer by size-exclusion chromatography with Sephadex G-25 (NAP™-25 column, Amersham Bioscience) and

HPLC (reversed-phase). The appropriate fractions were collected and analyzed.

Characterization of α-dPTP: α-Pyrene nucleoside triphosphate was prepared as previously described for the β isomer.[11,12a] The phosphorus NMR spectrum of α-dPTP in D_2O containing Tris-HCl (pH 7.5, 50 mM) and EDTA (2 mM) matched a previously published spectrum of β-dPTP, displaying a doublet at 9 ppm (α-phosphate), a doublet at 14 ppm (γ-phosphate), and a triplet at 25 ppm (β-phosphate).[12a] Electrospray mass spectrometry analysis of dPTP gave the expected mass (calcd for parent ion: 557.31; found: 557.1 $[M]^+$). The concentration of α-dPTP was calculated by using the extinction coefficient determined for 1-pyrenemethylamine hydrochloride at 276 nm in water.

Fluorescence methods: Fluorescence spectra were recorded on a Spex-Fluorolog-3 series fluorometer. The fluorescence measurements were taken in the right-angle mode on solutions in deionized water. The emission spectra were collected by using an excitation wavelength of 345 nm, and the excitation spectra were monitored at 375 nm. The excitation and emission slits were set to 3 nm. The spectra were normalized to have the same fluorescence intensity at their maximum wavelengths. Quantum yields were measured with fluorescein in NaOH solution (0.1 N) as a standard. The fluorescence image of the purified TdT reaction product and the β-dPTP monomers were taken while the solutions were illuminated with a 365 nm transilluminator.

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Keywords: apoptosis · excimers · fluorescent probes · pyrene · TUNEL

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Sensing Metal Ions with DNA Building Blocks: Fluorescent Pyridobenzimidazole Nucleosides

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Abstract: We describe novel fluorescent *N*-deoxyribosides (**1** and **2**) having 2-pyrido-2-benzimidazole and 2-quino-2-benzimidazole as aglycones. The compounds were prepared from the previously unknown heterocyclic precursors and Hoffer's chlorosugar, yielding alpha anomers as the chief products. X-ray crystal structures confirmed the geometry and showed that the pyridine and benzimidazole ring systems deviated from coplanarity in the solid state by 154° and 140°, respectively. In methanol compounds **1** and **2** had absorption maxima at 360 and 370 nm, respectively, and emission maxima at 494 and 539 nm. Experiments revealed varied fluorescence responses of the nucleosides to a panel of 17 monovalent, divalent, and trivalent metal ions in methanol. One or both of the nucleosides showed significant changes with 10 of the metal ions. The most pronounced spectral changes for ligand−nucleoside **1** included red shifts in fluorescence (Au^{+}, Au^{3+}), strong quenching (Cu^{2+}, Ni^{2+}, Pt^{2+}), and substantial enhancements in emission intensity coupled with red shifts (Ag^{+}, Cd^{2+}, Zn^{2+}). The greatest spectral changes for ligand−nucleoside **2** included a red shift in fluorescence (Ag^{+}), a blue shift (Cd^{2+}), strong quenching (Pd^{2+}, Pt^{2+}), and substantial enhancements in emission intensity coupled with a blue shift (Zn^{2+}). The compounds could be readily incorporated into oligodeoxynucleotides, where an initial study revealed that they retained sensitivity to metal ions in aqueous solution and demonstrated possible cooperative sensing behavior with several ions. The two free nucleosides alone can act as differential sensors for multiple metal ions, and they are potentially useful monomers for contributing metal ion sensing capability to DNAs.

Introduction

Although in nature DNA acts primarily as a storehouse of genetic information, recent studies have been aimed at endowing new properties on this genetic molecule. Among these properties is the ability to act as a sensor of physical conditions or molecular species. Because DNA has the innate ability to recognize other nucleic acids, many studies have reported on the use of modified DNAs as sensors of RNA and DNA.[1a−n] However, DNA (especially in the single-stranded form) can fold into diverse structures that allow for recognition of varied types of molecules,[2a−e] and many changes can be made to add functional groups of DNA to increase chemical diversity further.[3a−d] As a result, an increasing number of studies have focused on the use of modified DNA in detection of other species, such as peptides,[4] proteins,[5a,b] and small molecules,[6a−c] and sensing physical changes, such as pH[7] and light.[8] Few experiments have been aimed at sensing metal ions with DNAs as well.[9a−d] The properties of DNA as a sensor offer a number

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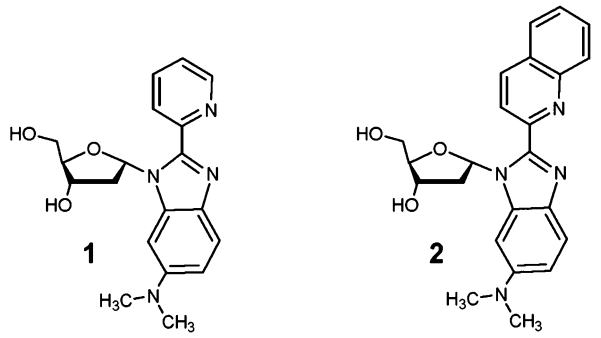
Chart 1. Structures 1 and 2



of advantages in application over other organic scaffolds; among these are water solubility, ease of synthesis (including automated methods), and predictable assembly and recognition properties.

As part of a larger program aimed at development of water-soluble, diverse fluorescent species, we adopted a strategy of replacing DNA bases with varied fluorescent groups.[10a−d] We considered the possibility that adding metal ligand functionality to fluorescent systems might render such species able to recognize and report on metal ions in solution. In this regard, a number of laboratories have recently described deoxyribosides with metal ligands as replacements for nucleobases,[11a−h] but these were designed for cross-linking nucleic acid strands, and use in ion detection was not reported.

Here we report on two new nucleosides in which the nucleobase is replaced by pyridobenzimidazoles that are designed to act as metal ligands. Their preparation, structure, and optical properties are described. The nucleosides are fluorescent and were found to be sensitive to the presence of 12 different metal ions, displaying significant changes in their emission spectra, both as free nucleosides and in the context of DNA. The compounds are expected to be useful in lending sensing properties to DNA.

Results

Nucleoside Design and Synthesis. Compounds **1** and **2** are bifunctional and possess both the structure of a DNA building block (i.e., deoxyribose) and structures designed to bind metals with conjugated systems (Chart 1). We chose these pyrido- (and related quino-) benzimidazole heterocycles (**3** and **4**), both of which were previously unknown, because simple pyridobenzimidazoles were previously known as ligands for metals,[12] because they have a rotatable bond that alters conjugation, and because they were known to be fluorescent. We added a dimethylamino group to the benzimidazole portion, placing this donor in conjugation with the pyridine ring nitrogen. Thus, the act of chelating a metal ion might be expected to alter conjugation by changing the dihedral angle of the single bond, which would alter the donation by the amino group. In addition, varied metals in the act of binding the ligand could also further alter the electronic states of the conjugated systems. Thus, we anticipated the possibility that these somewhat generic nitrogen ligands might yield diverse signals for different metal ions as a result of these variable electronic and structural factors.

We prepared **1** and **2** starting with commercial 5-chloro-2-nitroaniline (Scheme 1). The chloride was displaced by *N*,*N*-dimethylamine. Reduction of the nitro group to the amine followed by subsequent oxidative condensation with pyridine 2-carboxaldehyde in nitrobenzene yielded the heterocycle **3** and with quinoline 2-carboxaldehyde[13] yielded **4**. These were reacted with Hoffer's α-chlorosugar[14] in the presence of sodium hydride in *N*,*N*-dimethylacetamide. The chief products were assigned as α-anomers by proton NOE measurements. This stereochemical outcome is atypical for this reaction; we hypothesize that the steric bulk of the 2-aryl-benzimidazoles was better accommodated from the α face of the bis-toluoyl ester intermediate, yielding the α isomer in an S_N1 mechanism. Although there are two possible benzimidazole isomers for each nucleoside, we only observed the isomers in which the dimethylamino group is closer to the sugar; the origin of this preference is not yet clear but may be related to electron donation from this amino group into the imidazole ring. The ester protecting groups were subsequently removed under basic conditions. We were able to obtain single crystals of the free nucleosides **1** and **2** by crystallization from chloroform/methanol/hexane (Figure 1). The X-ray structures confirmed the α-anomeric geometry and showed that the pyridyl(quinolinyl) and benzimidazolyl rings deviated from syn coplanarity in the solid state by 154° and 140°, respectively. This apparent near-anti conformational preference may arise from avoidance of lone pair repulsions from the two nitrogens and/or possible steric repulsion of the pyridine protons away from the deoxyribose substituent.

Nucleoside Optical Properties. We examined the optical properties of the free nucleosides in solution. They were poorly soluble in water and dissolved in methanol and ethyl acetate for the measurements. In MeOH, both compounds showed small absorption maxima at 260 nm; **1** also had a major longer-wavelength absorption band at 360 nm, while **2** had an absorption at 370 nm (Table 1 and Supporting Information). Emission spectra were measured with excitation at the long-wavelength bands; they revealed fluorescence in the visible ranges for both compounds (Figure 2) with emission maxima at 494 and 539 nm for **1** and **2**, respectively. Quantum yields for these emission bands were 0.052 and 0.0085, respectively. The emission maxima in methanol were red shifted relative to the corresponding maxima in lower-polarity ethyl acetate solution (464 and 520 nm, respectively), consistent with

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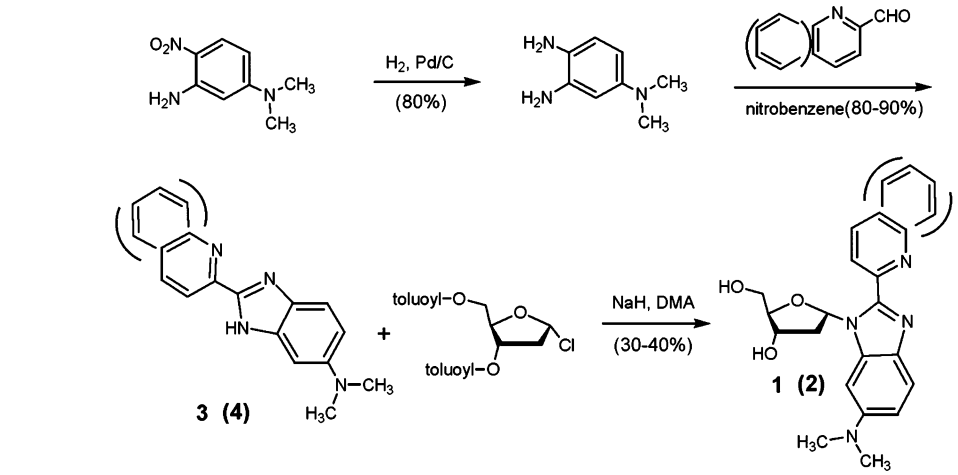
Scheme 1. Synthesis of Deoxyribosides 1 and 2



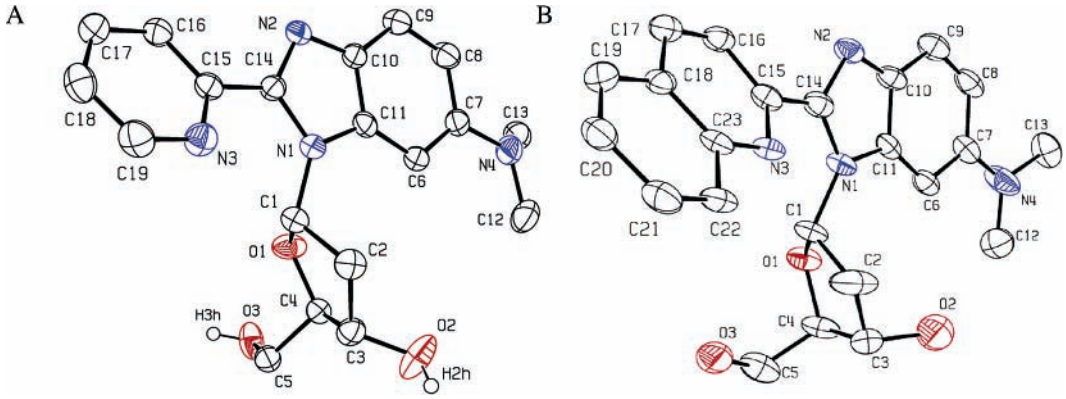


Figure 1. Structures of deoxynucleosides **1** (A) and **2** (B) in the solid state, determined by X-ray crystallography and shown in ORTEP form. Details are given in Supporting Information.

Table 1. Optical Data for Nucleosides 1 and 2

compd	solvent	absorbance max[a] (nm)	ϵ ($M^{-1}cm^{-1}$)	emission max (nm)	Φ_{fl}	τ (ns)[b]
1	EtOAc	360	nd	460	nd	nd
1	MeOH	360	1.37×10^4	494	0.050	0.45
2	EtOAc	370	nd	521	nd	nd
2	MeOH	384	1.36×10^4	543	0.0085	0.36

[a] Long-wavelength band is listed; shorter wavelength bands at 260 nm were also present. [b] Conditions: 10 μM nucleoside in methanol, 25 °C, with excitation at 370 nm. Compound. **1** was measured at 450 nm with a 430 nm UV filter; compound **2** was measured at 550 nm with a 515 nm UV filter.

formation of a strong excited-state dipole due to resonance overlap of the dimethylamino group with the pyridine ring nitrogen.

Nucleoside Response to Metal Ions. We then investigated the effects of metal ions on the fluorescence of nucleosides **1** and **2** in methanolic solution. Nucleosides and metals were studied at 50 and 100 μM concentrations, respectively, and we screened the effects of 17 metal species on the two nucleosides by monitoring emission spectra. Note that the ligands could in principle bind with varied stoichiometry with a given metal; some may bind as 1:1 ligand:metal complexes, while other combinations may form complexes with other stoichiometry. Metals (as their chloride salts) were added from concentrated stock solutions in DMSO, giving a final concentration of 5% DMSO in methanol. Results with compound **1** showed (Figure 3, Table 2, and Supporting Information) that a few metal ions (K^{2+}, Mg^{2+}, Ca^{2+}, Mn^{2+}, La^{2+}, Eu^{2+}) had little or no effect on the emission from the pyridobenzimidazole. Co^{2+} and Pd^{2+} showed weak quenching of this fluorophore, while Pt^{2+}, Cu^{2+}, and Ni^{2+} showed apparently strong quenching. In contrast, both Au^{+} and Au^{3+} gave marked red shifts in emission (shifts of 55 and 37 nm) with little effect on intensity. Finally, Ag^{+}, Cd^{2+}, and Zn^{2+} yielded both red shifts and marked increases in emission intensity; red shifts increased in the order Ag^{+} (26 nm shift with 3-fold increase in intensity) < Cd^{2+} (59 nm with 8-fold increase) < Zn^{2+} (79 nm with 3-fold increase). The effects are summarized in Table 2.

Metal ion effects on quinobenzimidazole **2** were also measured (Figure 4, Table 2, and Supporting Information) and revealed both similarities and differences with **1**. With **2**, eight metal ions (K^{+}, Mg^{2+}, Ca^{2+}, Hg^{2+}, Mn^{2+}, La^{2+}, Eu^{2+}) had little or no effect on the emission from ligand **1**. Co^{2+}, Au^{3+}, and Ni^{2+} showed slight quenching of this fluorophore, while Cu^{2+} showed moderate quenching in addition to a 5 nm red shift. Addition of Pd^{2+} and Pt^{2+} gave apparently strong quenching coupled with red shifts (shifts of 38 and 20 nm, respectively). In contrast, Ag^{+} gave a 36 nm red shift with little effect on intensity. Finally, unlike ligand **1**, ligand **2** yielded blue shifts of the emission band in two cases. Cd^{2+} yielded a shift from the original 543 nm emission to dual emission at 460 and 510 nm with little change in intensity, while Zn^{2+} yielded a shift to 476 nm and a substantial (2-fold) increase in emission intensity.

A summary of the results for both fluorescent nucleoside ligands is outlined in Table 2 and points out the similarities

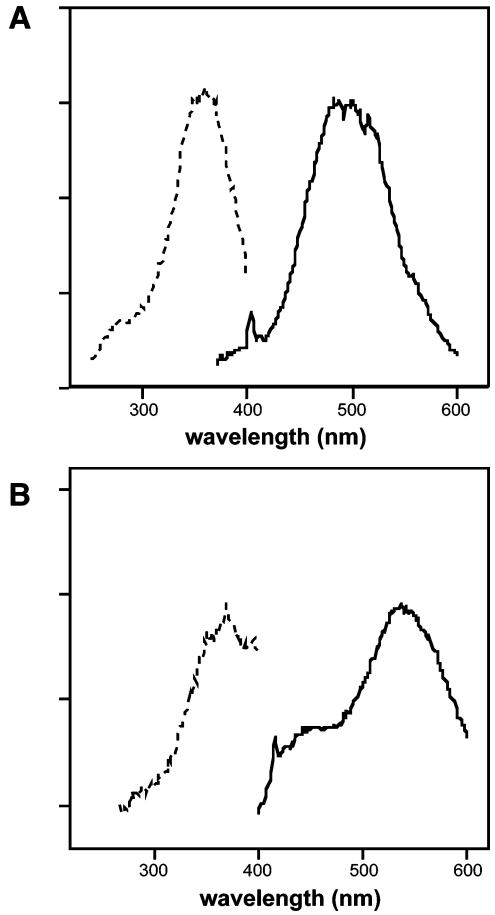


Figure 2. Excitation and emission spectra of **1** (A) and **2** (B) (2 μM) in methanol (25 °C). Excitation spectra are shown with dashed lines and were monitored at the emission maxima. Emission spectra are shown with solid lines and were measured with excitation at the long-wavelength absorption maxima.

and differences. As free nucleosides in methanol, neither compound showed significant changes with seven ions (K^{2+}, Mg^{2+}, Ca^{2+}, Hg^{2+}, Mn^{2+}, La^{2+}, Eu^{2+}). Both compounds showed similar changes for Co^{2+} (slight quenching) and Pt^{2+} (strong quenching with red shift). Interestingly, although they are similar ligands, the compounds showed different responses for the other eight metal ions. The most dramatic differences occurred with Ag^{2+} (>3-fold enhancement of fluorescence with **1** versus a red shift with **2**) and especially Zn^{2+} and Cd^{2+}, which showed marked red shifts and fluorescence enhancements with **1** but strong blue shifts with **2**.

Responses of Ligand Pairs in DNA. We then investigated the incorporation of fluorescent nucleosides **1** and **2** into DNA oligonucleotides. Both compounds were converted to the 5′-dimethoxytrityl-3′-phosphoramidite derivatives using standard methods (Supporting Information). They were then incorporated into two 13mer complementary oligonucleotides using automated synthesizer coupling programs. The presence of the intact ligands in the DNAs was confirmed by MALDI-TOF mass spectrometry. We also prepared complementary DNAs in which A, C, G, or T was paired opposite the ligands; these showed little or no differential effect on fluorescence emission (data not shown).

To investigate whether the fluorescence and recognition properties of these ligands were retained in water, we performed a screen of fluorescence responses of 13mer DNA duplexes in

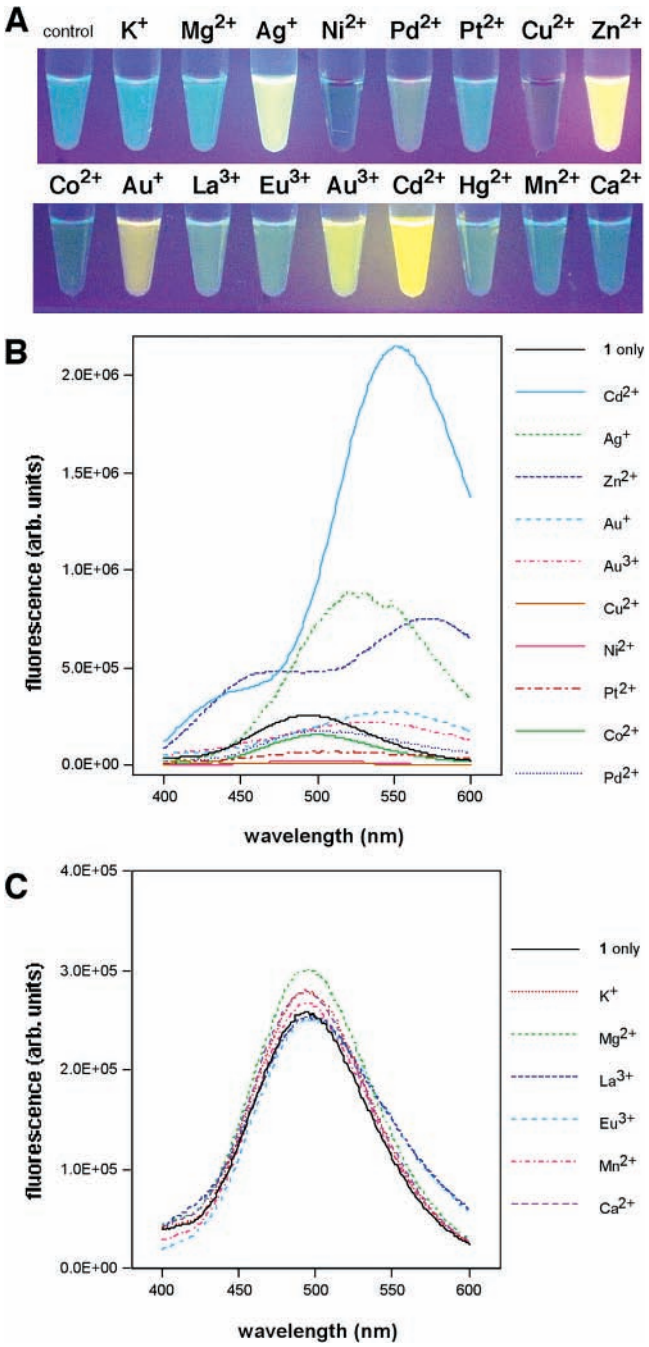


Figure 3. Fluorescence response of nucleoside **1** to 17 metal salts in methanolic solution (25 °C). (A) Photograph showing varied responses of **1** to the metals using 365 nm light from a UV box as the excitation source. (B) Spectra of **1** (2 μM) in the presence of metal ions that change the emission intensity or wavelength of the nucleoside. (C) Spectra of **1** with metal ions that yield little or no change in the emission properties. Metals are present at 100 μM as chloride salts except $AgNO_3$.

which nucleosides **1** and **2** were paired opposite themselves or each other (Figure 5). Four pairing geometries were tested: **1**−**1**, **2**−**2**, and the similar (but not identical) **2**−**1** and **1**−**2** cases. In the DNA context, nucleoside **1** (as a **1**−**1** pair) showed only a small change (a 5 nm red shift) compared to its behavior in methanol as a free nucleoside. Nucleoside **2**, on the other hand, showed a marked change with its major emission band appearing in the blue (440 nm) in DNA as a **2**−**2** pair, whereas alone in methanol **2** emits in the yellow range (543 nm). For the mixed **1**−**2** and **2**−**1** pairings, the emission behavior in the absence of

Table 2. Fluorescence Responses of Nucleosides 1 and 2 to Metal Salts in Methanolic Solution

compd	M^{n+}	emission max (nm)	intensity (rel)	compd	M^{n+}	emission max (nm)	intensity (rel)
1	none	494	1.0	2	none	543	1.0
1	K^+	494	1.1	2	K^+	544	1.1
1	Mg^{2+}	494	1.2	2	Mg^{2+}	543	1.1
1	Ca^{2+}	494	1.1	2	Ca^{2+}	541	1.0
1	Mn^{2+}	494	1.0	2	Mn^{2+}	540	1.0
1	Co^{2+}	494	0.6	2	Co^{2+}	539	0.9
1	Ni^{2+}	496	0.1	2	Ni^{2+}	542	0.9
1	Cu^{2+}	486	0.1	2	Cu^{2+}	548	0.6
1	Zn^{2+}	573	2.9	2	Zn^{2+}	476	2.0
1	Pd^{2+}	503	0.7	2	Pd^{2+}	581	0.2
1	Ag^+	520	3.4	2	Ag^+	579	1.0
1	Cd^{2+}	553	8.3	2	Cd^{2+}	510	1.2
1	Pt^{2+}	508	0.3	2	Pt^{2+}	563	0.3
1	Au^+	549	1.1	2	Au^+	577	0.8
1	Au^{3+}	531	0.9	2	Au^{3+}	552	0.8
1	La^{3+}	498	1.0	2	La^{3+}	552	1.0
1	Eu^{3+}	496	1.0	2	Eu^{3+}	548	1.0
1	Hg^{2+}	498	0.9	2	Hg^{2+}	540	0.8

[a] Conditions: 50 μM nucleoside, 100 μM metal ion, 95% methanol, 5% DMSO, 25 °C. All counterions were chloride except $AgNO_3$.

metals resembled that of the **2**−**2** pairing more than the **1**−**1** pairing (Supporting Information), suggesting that **2** may quench the emission of **1**. However, in the presence of metals, the responses were more similar to those of the **1**−**1** pairing (see below).

We then screened these four different ligand "base pairs" for their responses to the addition of metal ions (Figure 5). In some cases we found roughly parallel responses to those seen for the free nucleosides in methanol; for example, the **1**−**1** pairing showed strong quenching by Pd^{2+} and Pt^{2+} and displayed enhancements of fluorescence and red shifts with Zn^{2+} and Cd^{2+}, similar to what was observed for **1** alone in methanol. In another example Cd^{2+} gave essentially the same results whether with **2** alone or with the **2**−**2** pairing in DNA. For the mixed **1**−**2** and **2**−**1** pairings, the emission behavior in the presence of metals resembled the results with the **1**−**1** pairing more than the **2**−**2** pairing.

However, there were some marked differences between the fluorescence emission behavior in the DNA setting and that of the ligand nucleosides alone. For ligand **1**, Cd^{2+}, Ag^+, and Zn^{2+} yielded considerably greater enhancements of fluorescence with the **1**−**1** pairing in DNA, giving 20-, 15-, and 11-fold overall enhancements, respectively, in emission intensity. Au^{3+} yielded a red shift in methanol, whereas with **1**−**1** in DNA it quenched fluorescence. In another example Pd^{2+} did not quench **1** alone, whereas it strongly quenched **1**−**1** in DNA. For ligand **2**, Zn^{2+} yielded a new band in the red for the **2**−**2** pairing in DNA, whereas alone in methanol it gave enhancement plus a blue shift. The Ag^+ ion yielded dual bands at 440 and 552 nm with **2**−**2** in DNA, whereas with **2** in methanol it yielded only a 36 nm red shift.

In a few other cases the ligand−nucleosides showed additional striking evidence of apparently cooperative behavior in a "paired" configuration (see Figure S2 in Supporting Information). For example, the **1**−**1** pairing yielded enhanced signals in the presence of Eu^{3+}, whereas **1** alone in methanol showed little or no effect with this lanthanide. In a second example La^{3+} yielded a strong change for the **2**−**2** pairing with a new red band appearing at 541 nm, whereas **2** alone in

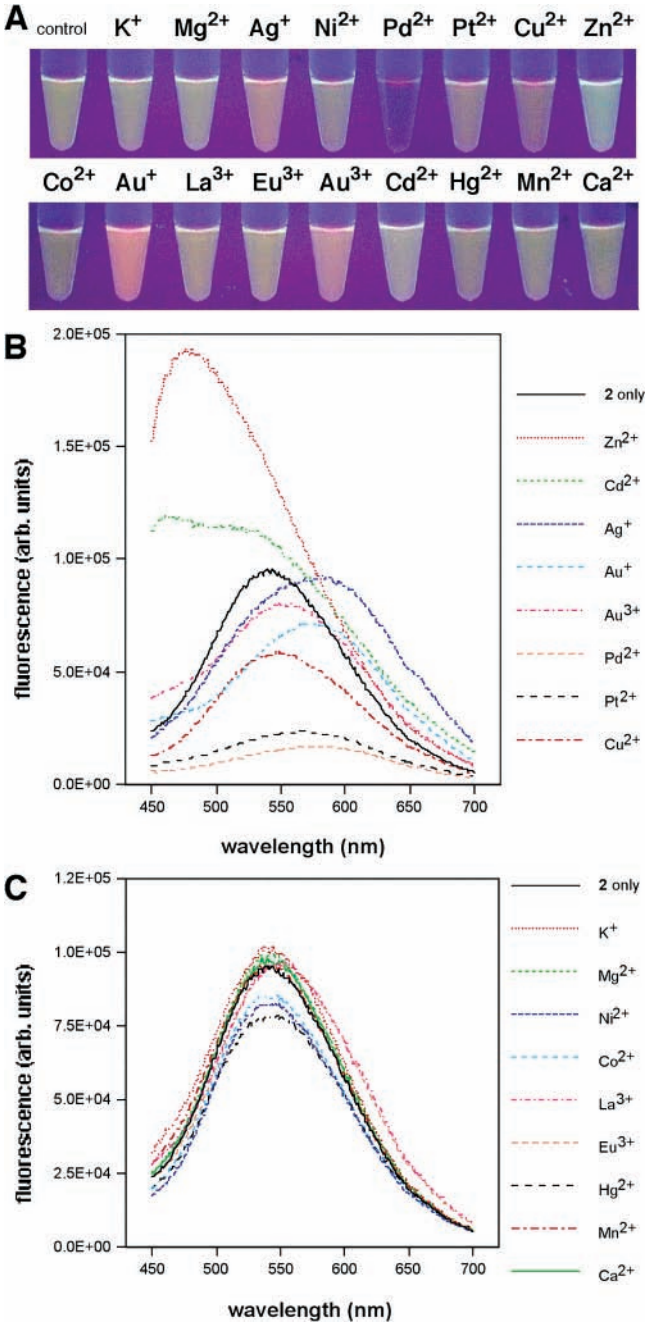


Figure 4. Fluorescence response of nucleoside **2** to 17 metal salts in methanolic solution (25 °C). (A) Photograph showing varied responses of **2** to the metals using 365 nm light from a UV box as the excitation source. (B) Spectra of **2** (2 μM) in the presence of metal ions that change the emission intensity or wavelength of the nucleoside. (C) Spectra of **2** with metal ions that yield little or no change in the emission properties. Metals are present at 100 μM as chloride salts except $AgNO_3$.

methanol yielded no change with this ion. In a third example the **2**−**1** pairing showed a nonquenched red shift with Ni^{2+}, whereas this metal strongly quenched **1** alone in methanol and had no effect on **2**. Finally, Hg^{2+}, another ion to which neither **1** nor **2** responded alone, yielded strong quenching in DNA containing the **2**−**2** pairing.

Discussion

These early experiments show that combinations of one or both of these nucleoside ligands, either as free nucleosides or in DNA, could sense 12 of the 17 metal ions in this initial panel

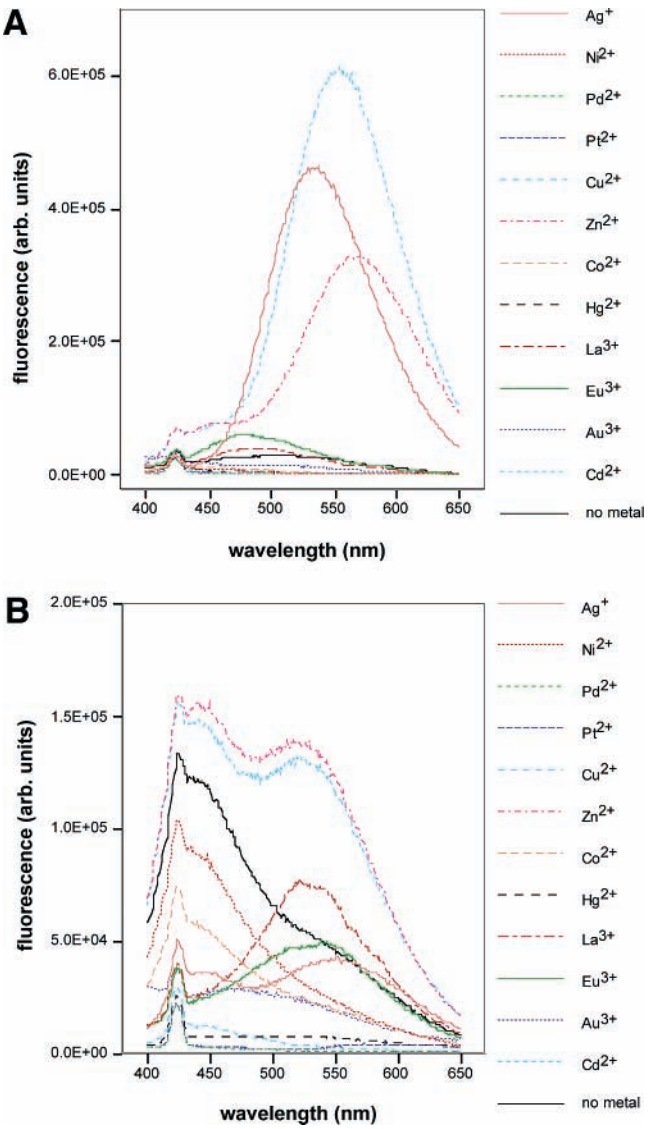


Figure 5. Fluorescence responses of **1** and **2** to metal ions in aqueous solution, measured in the context of 13mer duplex DNAs having ligands **1** and/or **2** placed opposite each other, as pseudo base pairs. (A) Emission spectra of DNA with nucleoside **1** paired opposite itself in the presence of varied metal ions. (B) Emission spectra of DNA with ligand **2** paired opposite itself. See Figure S2 (Supporting Information) for cases with ligand **2** paired with **1**. Solutions contained 3 μM DNA duplex and 100 μM metal ion at 25 °C. Buffers also contained 100 mM NaCl and 10 mM Na·PIPES (pH 7.0). Excitation was at 370 nm.

of salts and allow one to distinguish between all 12 (except Pd^{2+} and Pt^{2+}, which give similar quenching effects with both ligands). We expect that the diverse responses arise from the combination of the rotatable bond, the electron-donating amino group in conjugation with the pyridine nitrogen, and variable electronic interactions of the ligands with the individual metals. Further experiments will be needed to understand the details of these mechanisms for the individual ligand−ion combinations.

With the current ligand design, which employs bipyridyl-like structure, it is not surprising that the fluorescence responses are weak or absent for the more oxophilic metals in this panel of analytes, such as K^+, Mg^{2+}, Ca^{2+}, Hg^{2+}, and Mn^{2+}. These metals are expected to have weak affinity for the current nitrogen ligands; future nucleoside designs for sensing these species may require different liganding groups. However, our data for the **2**−**2** pairing in DNA show that this combination of two ligands can apparently act cooperatively to recognize and give a response signal for Hg^{2+}. Beyond the current studies, we do expect that the current nucleosides may be able to respond to the presence of other cations not included in this initial panel, and future studies will address this. One limitation should be noted for these nucleosides, namely, that they are only weakly water soluble and thus limited in their aqueous applications unless organic cosolvent can be added. Future studies will address whether more polar derivatives can be prepared. Of course, in the DNA context (see below) water solubility is no longer problematic.

As a whole, the fluorescence responses of nucleosides **1** and **2** are remarkably diverse. Nucleoside **1** in methanol gives emission maxima over an 87 nm wavelength range and a >80-fold intensity range depending on the metal ion. Nucleoside **2** gives a similar 85 nm emission wavelength range, yielding a red-shifted emission with Ag^+ and a blue-shifted emission with Cd^{2+}. Overall, ligand **1** gives at least five qualitatively different responses (strong quenching, weak quenching, strong emission enhancement, red shift, multiple emission bands) for different ions, and ligand **2**, differing from **1** only by benzo substitution, also gives at least five qualitatively different responses, in some cases markedly different than **1**. Taken together, the data suggest that monomer **1** can act as a useful "turn on" sensor of Ag^+, Cd^{2+}, Zn^{2+}, and Au^+ ions, and the responses to these are different enough to allow separate identification of these metals in an unknown sample. Because the responses to Au^+ and Au^{3+} are similar, **1** would not be useful in discriminating between the latter two. In the quenching mode both Ni^{2+} and Cu^{2+} give very strong quenching of this nucleoside, allowing one to discriminate between this pair and the above fluorescence-enhancing ions. Monomer **2**, like **1**, can also be used as enhanced-signal reporters of Zn^{2+} and Cd^{2+}, and it also gives a marked positive response for Ag^+ and Au^+, although it is difficult to distinguish between the last two. Unlike **1**, **2** can distinguish between Au^+ and Au^{3+}, by a 25 nm difference in wavelength. In quenching applications ligand **2** is not as strongly quenched by the metals in this panel as **1** and would not be as useful as the latter ligand.

In addition to fluorescence responses involving an increase or a decrease in intensity, these ligand nucleosides in a number of cases change their emission wavelengths markedly in the presence of some metal ions. This is especially useful for two reasons: first, it allows for ratiometric measurements of concentration, as the ratio of two wavelengths can easily be measured. Second, it allows for distinguishing between background signal and positive signal in applications where separating the reporter from the analyte is undesirable or impossible, such as in real-time solution-phase assays and intracellular assays. Nucleoside **1** shows its most marked red shifts in wavelength for Cd^{2+} (59 nm), Zn^{2+} (79 nm), and Au^+ (54 nm) and thus might in principle be used ratiometrically for these. Nucleoside **2** shows the greatest shifts for Zn^{2+} (67 nm) and Cd^{2+} (33 nm) (these are blue shifts rather than red shifts).

A comparison of the fluorescence responses of **1** and **2** with other recently reported sensors reveals some similarities and differences. There exist a substantial number of reports of

sensors for Zn^{2+},[15] including some that respond with a single wavelength and a few that give a change in wavelength,[15b,d,g] which is useful for ratiometric measurements. Compounds **1** and **2** fall into the latter category; it is not yet clear whether they would be useful in detecting Zn^{2+} in cellular media as their cell permeability and affinity for low biological concentrations of this ion are not yet known. Compounds **1** and **2** also detect Cd^{2+}, Au^{+}, and Ag^{+} with enhancements of emission and wavelength shifts; although Zn^{2+} sensors often respond to Cd^{2+} as well, compound **1** is unusual in that it can readily distinguish between these two closely related ions by wavelength. Finally, we have shown that base pairs involving these nucleosides can also respond to lanthanides with wavelength shifts. Thus, although metal ion sensing (especially of Zn^{2+}) is by now well established in the literature, the diversity of responses of these two nucleoside−ligands with a range of metal ions is rare.

Our data show that these nucleosides can be readily used as monomers in the synthesis of DNA and that they retain metal ion recognition properties in aqueous buffers and in the context of the DNA double helix. We have shown that pairing of two nucleoside ligands opposite one another can in some cases yield new recognition and sensing properties that the monomers alone do not have. A number of laboratories have described the strategy of assembling base pairs in which a metal ion cross links two paired ligands as DNA base replacements.[11a−h] The current studies build on that concept by showing a fluorescence reporting response as a result of that apparent interaction. Examples of responses that may be specially useful in application are those of the **1**−**1** pairing, which gives strong fluorescence enhancements and red shifts with Cd^{2+}, Ag^{+}, and Zn^{2+}; it is even possible to distinguish between the three by wavelength. The **2**−**2** pairing may also be especially useful in sensing of Cd^{2+} and Zn^{2+}, although these cannot be distinguished from one another. More useful may be the application of **2**−**2** in sensing of Ag^{+} and La^{3+}, where the original blue short-wavelength band is strongly quenched and new long-wavelength yellow bands appear. These findings, coupled with those of the nucleosides alone, suggest that DNAs containing **1** and **2** might have useful ion sensing applications in both single-stranded and double-stranded forms. Future studies will investigate this in detail.

In summary, the present compounds show promise not only as sensors alone but also as components for introducing metal ion sensing capability into DNA. There is now a broad number of literature reports describing fluorescence sensing of metal ions by ligands in solution,[15,16] but few if any of these are directly adaptable to DNA.[16h] We anticipate that the use of DNA as a scaffold for this fluorescence sensing will be advantageous by adding aqueous solubility to these heterocyclic species, thus allowing possible applications in biological systems and aqueous waste streams. In addition, the use of DNA double-helical or folded structure may allow for useful cooperative sensing between multiple ligands as our preliminary studies suggest. Finally, the DNA portion of such molecules could be useful for self-assembly of sensors on surfaces and in arrays.

Experimental Section

Nucleoside Synthesis. 5-*N*,*N*-Dimethylamino-2-(2-pyridinyl)-1*H*-benzimidazole (**3**).

Nitrobenzene (200 mL) was added to 4-*N*,*N*-(dimethylamino)-phenylenediamine (19.2 mmol) to make a solution in a round-bottom flask. 2-Pyridinecarboxaldehyde (1.83 mL, 19.2 mmol) was added, and then the solution was slowly heated to 100 °C. After 1 h stirring, the reaction mixture was further heated to 150 °C and stirred for an additional 2 h. This solution was cooled to room temperature and directly loaded on a silica gel column for chromatographic purification (eluent CH_2Cl_2/hexane (1:1) followed by CH_2Cl_2/EtOAc (2:3)). The product was obtained as a dark yellow powder (3.57 g, 15.0 mmol, 78% yield over two steps). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.64 (d, J = 5.2 Hz, 1H), 8.20 (d, J = 8.0 Hz, 1H), 7.92 (t, J = 8.0 Hz, 1H), 7.46−7.40 (m, 2H), 6.82−6.78 (m, 2H), 2.88 (s, 6H). ^{13}C NMR (100.7 MHz, DMSO-d_6): δ 149.9, 149.8, 149.3, 148.8, 138.1, 124.8, 121.5, 112.1, 96.9, 41.9. HRMS (DEI): $[M]^+$ calcd for $C_{14}H_{14}N_4$, 238.1218; found, 238.1226.

5-*N*,*N*-Dimethylamino-2-(2-quinolinyl)-1*H*-benzimidazole (**4**). Nitrobenzene (250 mL) was added to 4-*N*,*N*-(dimethylamino)phenylenediamine (27.1 mmol) to make a solution in a round-bottom flask. 2-Quinolinecarboxaldehyde (4.14 g, 26.3 mmol) was added, and then the solution was slowly heated to 80 °C. After 1 h stirring, the reaction mixture was further heated to 150 °C and stirred for an additional 24 h. This solution was cooled to room temperature and directly loaded onto a silica gel column for chromatographic purification (eluent CH_2Cl_2/hexane (1:1) followed by CH_2Cl_2/EtOAc (1:1)). The product was obtained as an orange yellow powder (4.86 g, 16.9 mmol, 62% yield over two steps). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.56 (d, J = 8.8 Hz, 1H), 8.25 (d, J = 8.4 Hz, 1H), 8.09 (d, J = 8.4 Hz, 1H), 7.83 (d, J = 8.0 Hz, 1H), 7.71−7.65 (m, 2H), 7.51 (t, J_1 = 7.3 Hz, 1H), 6.81 (dd, J_1 = 9.2, J_2 = 2.4 Hz, 1H), 6.28 (s, 1H), 2.84 (s, 6H). ^{13}C NMR (100.7 MHz, $CDCl_3$): δ 149.4, 149.2, 149.1, 147.8, 137.4, 137.3, 136.0, 129.1, 128.5, 128.1, 127.1, 120.6, 119.4, 111.6, 93.5, 41.5. HRMS (DEI): $[M]^+$ calcd for $C_{18}H_{16}N_4$, 288.1375; found, 288.1375.

1-(5-*N*,*N*-Dimethylamino-2-(2-pyridinyl)benzimidazole)-1′-α-deoxyriboside (**1**). 5-*N*,*N*-Dimethylamino-2-(2-pyridinyl)-1*H*-benzimidazole (1.1 g, 4.54 mmol) and NaH (0.20 g, 4.99 mmol) were charged into a 100 mL round-bottom flask. A 15 mL amount of *N*,*N*-dimethylacetamide was added into the flask via syringe under an atmosphere of argon. The reaction mixture was stirred at room temperature for 30 min, then Hoffer's chlorosugar (2.12 g, 5.39 mmol) was added in one portion, and the reaction mixture was stirred for 2 h at room temperature. The solvent was evaporated under vacuum. The crude mixture was extracted with water and CH_2Cl_2. The organic layer was dried with Mg_2SO_4 and evaporated under reduced pressure. The crude product was dissolved in 30 mL of CH_2Cl_2/MeOH (1:1), and then 4.5 mL of NaOMe (0.5 N in MeOH) was added into the flask via syringe under an atmosphere of argon. The reaction mixture was stirred at room temperature for 1 h. The solvent was evaporated under reduced pressure. The crude

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products were purified by silica gel chromatography (eluting with hexane/ethyl acetate 1:1 to 1:3) to give the desired product (562 mg, 1.59 mmol, 35% yield). ^{1}H NMR (400 MHz, $CDCl_3$/ CD_3OD (1:1)) δ 8.65 (d, $J = 4.8$ Hz, 1H), 8.03 (d, $J = 8.0$ Hz, 1H), 7.90 (td, $J_1 = 8.0$, $J_2 = 2.0$ Hz, 1H), 7.56 (d, $J = 8.4$ Hz, 1H), 7.41 (dd, $J_1 = 7.6$, $J_2 = 5.2$ Hz, 1H), 7.34 (d, $J = 2.4$ Hz, 1H), 7.12 (t, $J = 8.0$ Hz, 1H), 6.94 (dd, $J_1 = 9.2$, $J_2 = 2.4$ Hz, 1H), 4.55−4.50 (m, 1H), 4.31−4.27 (m, 1H), 3.75 (dd, $J_1 = 12.0$, $J_2 = 3.6$ Hz, 1H), 3.65 (dd, $J_1 = 12.0$, $J_2 = 4.8$ Hz, 1H), 3.02 (s, 6H), 2.96−2.88 (m, 1H), 2.72−2.65 (m, 1H). ^{13}C NMR (100.7 MHz, $CDCl_3$/CD_3OD (1:1)): δ 150.0, 149.1, 148.9, 148.8, 137.5, 135.2, 134.6, 125.1, 124.1, 119.5, 112.4, 96.9, 87.2, 86.8, 71.0, 62.2, 41.4, 39.2. MS (EI): $m/z = 355.4$ $[M + H]^+$. HRMS (DCI): $[M + H]^+$ calcd for $C_{19}H_{23}N_4O_3$, 355.1770; found, 355.1780.

1-(5-*N*,*N*-Dimethylamino-2-(2-quinolinyl)benzimidazole)-1′-α-deoxyriboside (**2**). 5-*N*,*N*-Dimethylamino-2-(2-quinolinyl)-1*H*-benzimidazole (1.13 g, 3.9 mmol) and NaH (264 mg, 6.6 mmol) were charged into a 100 mL round-bottom flask. A 20 mL amount of *N*,*N*-dimethylacetamide was added into the flask via syringe under an atmosphere of argon. The reaction mixture was stirred at room temperature for 30 min; then solvent was evaporated under vacuum. The crude mixture was extracted with water and CH_2Cl_2. The organic layer was dried with Mg_2SO_4. The crude products were purified by silica column chromatography (eluting with hexane/ethyl acetate/dichloromethane 2:1:2 to 1:1:1) to give the desired product (971.4 mg, 1.52 mmol, 39% yield). This bis-toluoyl-protected deoxyriboside (971.4 mg 1.52 mmol) was dissolved in 50 mL of CH_2Cl_2/MeOH (1:1), and then 10 mL of NaOMe solution (0.5 N in MeOH) was added into the flask via syringe under an atmosphere of argon. The reaction mixture was stirred at room temperature for 1 h. The solvent was then evaporated under reduced pressure, and the crude products were purified by silica column chromatography (eluting with hexane/ethyl acetate 1:1 to 1:3) to give the desired product (541 mg, 1.34 mmol, 88% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.45 (d, $J = 8.8$ Hz, 1H), 8.30 (d, $J = 8.8$ Hz, 1H), 8.06 (d, $J = 8.4$ Hz, 1H), 7.99 (d, $J = 7.6$ Hz, 1H), 7.80 (t, $J = 8.0$ Hz, 1H), 7.66−7.55 (m, 3H), 7.33 (s, 1H), 6.88 (dd, $J_1 = 8.8$, $J_2 = 2.4$ Hz, 1H), 5.63 (br, 1H), 4.95 (t, $J = 5.2$ Hz, 1H), 4.45 (br, 1H), 3.58−3.47 (m, 2H), 3.04−2.93 (m, 7H), 2.49−2.43 (m, 1H). ^{13}C NMR (100.7 MHz, DMSO-d_6): δ 150.7, 148.7, 148.3, 147.1, 137.6, 135.9, 135.7, 131.0, 129.8, 128.6, 128.1, 127.7, 122.3, 120.6, 112.0, 96.8, 88.2, 87.3, 71.4, 62.5, 41.6, 39.2. MS (EI): $m/z = 405.2$ $[M + H]^+$. HRMS (DCI): $[M + H]^+$ calcd for $C_{23}H_{25}N_4O_3$, 405.1927; found, 405.1924.

Oligonucleotide Synthesis. We prepared the 5′-O-DMTr-protected 3′-2-cyanoethyl phosphoramidite building blocks of **1** and **2** following standard methods (see Supporting Information). We directly applied them using solid-phase oligodeoxynucleotide (ODN) synthesis protocols on an automated DNA synthesizer (Applied Biosystems 394 DNA/RNA synthesizer). All oligodeoxynucleotides were synthesized on 1.0 μmol scale using standard β-cyanoethyl phosphoramidite chemistry but with extended (200 s) coupling times, double coupling, and high concentration (0.13 mM) for modified nucleosides. Stepwise coupling yields for nonnatural nucleosides were all greater than 95% as determined by trityl monitoring. The synthesized oligonucleotides were cleaved from the solid support by treatment with 30% aqueous NH_4OH (1.0 mL) for 10 h at 55 °C. The crude products from the automated ODN synthesis were lyophilized and diluted with distilled water (2 mL). The ODNs were purified by HPLC (Zorbax ODS column, 9.4 × 250 mm). The fractions containing the purified ODN were pooled and lyophilized. Aqueous AcOH (80%) was added to the ODN for deprotection of the 5′-terminal DMT. After 30 min at ambient temperature, the AcOH was evaporated under reduced pressure. The residue was diluted with water (2 mL), and the solution was purified by HPLC again. Modified oligonucleotides in the study were characterized by matrix-assisted laser-desorption-ionization time-of-flight (MALDI-TOF) mass spectrometry (see Supporting Information).

Fluorescence Measurements. Fluorescence measurements were carried out on a Spex Fluorolog 3 fluorescence spectrometer equipped with a Lauda Brinkmann RM 6 temperature controller. Fluorescence quantum efficiency (ϕ_{samp}) was obtained by comparing the integrated fluorescence spectra of a sample (I_{samp}) with that of a reference sample (I_{ref}) having a known fluorescence quantum efficiency (ϕ_{ref}). We used 9,10-diphenylanthracene (10 μM) dissolved in EtOH ($\phi_{ref} = 0.81$, excitation at 366 nm) as a standard. Compounds **1** and **2** were prepared as 10 μM solutions in methanol. Quantum yields were calculated by the following equation

$$\phi_{samp} = \phi_{ref} \times (A_{ref}/A_{samp})(\eta_{samp}/\eta_{ref})^2(I_{samp}/I_{ref})$$

Absorption spectra were measured with a Cary I UV−vis spectrometer, and excitation and emission spectra were observed by a Spex Fluorolog 3 fluorescence spectrometer. Fluorescence lifetimes were measured on a Photon Technologies, Inc. Easylife LS instrument; a 465 nm pulsed LED was utilized for excitation, and a 535 nm long-pass filter selected the desired emission wavelengths.

Screening of Metal Ions. Seventeen metal salts were tested for fluorescence responses with **1** and **2**: KCl, $MgCl_2$, $AgNO_3$, $NiCl_2$, $PdCl_2$, $PtCl_2$, $CuCl_2$, $ZnCl_2$, $CoCl_2$, AuCl, $LaCl_3$, $EuCl_3$, $AuCl_3$, $CdCl_2$, $HgCl_2$, $MnCl_2$, $CaCl_2$. All metal salt solutions were prepared as stock solutions (5.0 mM) in DMSO. Note that AuCl had poor solubility and left some precipitate. Nucleosides **1** and **2** were separately prepared as 50 μM solutions in methanol, and each metal salt stock solution was added to give 2-fold excess metal concentration (100 μM). Photographs were taken over a UV light box (354 nm), and fluorescence spectra were measured as described above.

Crystal Structure Determination. Single crystals of free ligand−nucleosides **1** and **2** were grown by a bilayer method. The compounds were dissolved in a 1:1 mixture of dry $CHCl_3$ and MeOH. These solutions (ca. 1 mL) were divided to NMR tubes (5 × 200 mm), and hexane (ca. 2 mL) was added very slowly, making a second layer. At the junction between the sample layer (lower) and the hexane (upper) layer we obtained crystals for X-ray diffraction. The X-ray data for **1** and **2** were collected on a Siemens SMART-APEX CCD single-crystal X-ray diffractometer. The structures were solved and refined by full-matrix least-squares techniques on F^2 using SHELX-97. Additional details are in the Supporting Information.

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Supporting Information Available: Details of the synthesis and characterization of phosphoramidite derivatives of **1** and **2**; characterization of modified oligonucleotides; fluorescence data. This material is available free of charge via the Internet at http://pubs.acs.org.

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